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A total of 16 selected compounds which included some natural products as well as drugs known to be efficacious against other diseases were studied at various dosage levels and via various routes of administration in hamsters for antileishmanial efficacy against visceral leishmaniasis (Leishmania Leishmania donovani) and three of the same compounds were studied for activity against cutaneous leishmaniasis (Leishmania Vianni panamensis). None of these were efficacious against either leishmania but several were toxic to the host.

Leishmanial cutaneous lesion development in golden hamsters is very helpful, along with other procedures, for the confirmation of the presence of Leishmania in cultures obtained by WRAIR from patients with suspected infections with these parasites.

The infection of golden hamsters with several species of Leishmania of human origin (identified in other laboratories by biochemical typing as Leishmania Viannia panamensis, Leishmania Leishmania mexicana, and Leishmania Viannia braziliensis) resulted in clinical disease similar to that seen in human beings. Thus a laboratory animal model is now in place for the performance of drug sensitivity studies on these new isolates of Leishmania, some of which apparently had various degrees of susceptibility to current therapeutic drugs.

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William L. Hanson — April 18, 1997

PI - Signature — Date

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Introduction

Protozoan parasites of the genus Leishmania are widespread throughout the world with at least 80 countries known to be endemic for these parasites where they cause a complex of visceral or cutaneous diseases in human beings as well as some domestic animals such as dogs (1-3). Since the leishmaniases commonly exist as zoonoses, these diseases pose a significant potential threat to the health and performance of military personnel as well as military dogs throughout the extensive endemic areas throughout the world (1, 4-7). Relatively recent publicity in both scientific and other media regarding possible infection with these diseases of personnel involved in Operation Desert Storm has reemphasized the military significance of the leishmaniases. Available current information regarding the importance of leishmaniasis as an opportunistic infection in HIV-infected persons living in both endemic and non-endemic areas (8, 9) adds to the importance of these parasites in human health.

The leishmaniases are extremely difficult to treat in general and treatment of patients also infected with HIV is especially difficult (10, 11). Although chemotherapy offers the best potential mechanism for the management of these diseases in infected human beings or animals, drugs currently available for use against these parasites are often not satisfactorily effective and are potentially toxic to man and animals. Consequently, better orally administered drugs for the treatment of the leishmaniases are much needed.

As a result of the importance of Leishmania to human and animal health, this laboratory has been involved for several years in studies to identify new antileishmanial compounds which are more efficacious against these parasites and less toxic for laboratory animals and hopefully for human beings. In these studies we have refined animal model systems for testing potential compounds with antileishmanial efficacy using both visceral (Leishmania Leishmania donovani) and cutaneous (Leishmania Viannia braziliensis) leishmaniasis in hamsters as well as non-human primates (12-16).

While some success has been achieved in the search for better antileishmanial drugs in these studies (17-21), as well as by studies of others (22), most of these compounds have met with limited success in the field in both immunocompetent and immunocompromised patients. Thus it is necessary to continue the search for better antileishmanial drugs.

Because of the need for better antileishmanial drugs for use in humans and dogs, the primary purpose of this project is to receive potential antileishmanial drugs from officials at the Walter Reed Army Institute of Research (WRAIR) and test these compounds for efficacy against Leishmania in rodents as well as non-human primates when warranted. During part of this project period this laboratory has also received cultures obtained by officials at WRAIR from patients suspected of being infected with Leishmania (or possibly some other blood parasite) with two primary objectives: (a) verification of leishmanial infections by injection of these cultures primarily into hamsters and observing any clinical disease manifestations that developed or by subinoculation of the cultures into other media; (b) determination of the infectivity of these Leishmania for hamsters and obtain preliminary data regarding infections observed thus enabling future in vivo drug sensitivity studies of these isolates in laboratory animals. Data obtained from these studies will be important for the future management of leishmanial infections in patients at WRAIR as well as other patients elsewhere.

Materials and Methods

I. Testing of Potential Antileishmanial Compounds

A. Primary Visceral Test System

A Khartoum strain of Leishmania (L.) donovani (WR378) was used and the golden hamster (Mesocricetus auratus, Harlan Sprague Dawley, Inc., Indianapolis, Indiana), 50-70 gm, served as the host animal. Suspensions of amastigotes for infection of experimental hamsters were prepared by grinding heavily infected hamster spleens in sterile saline in a Ten Broeck tissue grinder and diluting the suspensions so that 0.2 ml contained approximately 10 X 10^6 amastigotes. After being tranquilized with 0.3 ml of a Ketamine Hydrochloride/Rompun (Xylazine) solution (16.7 mg/ml and 5.0 mg/ml respectively) each experimental hamster was infected via the intracardiac injection of 0.2 ml of the amastigote suspension.

The testing procedure used was that described by Stauber and his associates (23, 24) as modified by Hanson, et al. (12). On Day 3 following infection, hamsters were divided randomly into experimental groups consisting of a minimum of 6 animals per group, initial group weights were obtained, and administration of test compounds was initiated. Each compound was tested at 2 or 3 drug dosage levels dependent upon the priority rating and nature of the compound.

The vehicle for the test compounds was 0.5% hydroxyethyl cellulose-0.1% Tween 80 (HEC-Tween). Each test group contained six hamsters and received one of the desired drug dosage levels. A control group of six hamsters received the 0.5% HEC-Tween vehicle only and the reference compound, Glucantime®, was given at 3 drug dosage levels (208, 52, and 26 total mg/kg) based on antimony content. Test compounds were administered routinely once daily via various routes dependent on the nature of the compound on Days 3 through 6. Final group weights were obtained on all experimental hamsters on Day 7 and all animals were killed with CO_2, livers removed, weighed, and liver impressions made for enumeration of amastigotes. Subsequently, the total number of parasites per liver was determined as described by Stauber, et al. (23. 24).

In addition to recording body weight changes as a general indicator of toxicity of the test compounds, experimental hamsters were observed for such clinical signs of toxicity as nervous disorders, roughened hair coat, and

sluggish activity. Deaths of the animals were also used as an indication of significant drug toxicity.

After determining the ratio of numbers of amastigotes per host cell nucleus, the weight of the organ, and initial and final weights of the hamsters, the raw data was evaluated with a Gateway 2000 microcomputer using a program which calculates percent weight change, total numbers of parasites, mean numbers of parasites per organ, and percent parasite suppression. The computer program then performs linear and non-linear regression analysis and calculates a SD_{50} for active compounds from each of the analyses (drug dosage resulting in 50% suppression of amastigotes). The SD_{50} from the non-linear analysis is used for a comparison of the relative efficacies of the test compounds as well as the relative efficacy of test compounds to that of the reference compound, Glucantime. The linear regression analysis is included only for comparison with the non-linear analysis.

B. Primary Cutaneous Test System

Leishmania (V.) braziliensis (WR539) was used in these studies. Male golden hamsters, 50-70 gm, served as experimental hosts.

Promastigotes for establishing experimental infections in hamsters were grown in Schneider's Drosophilia Medium (Hendricks, et al., 25) and quantitated using procedures described previously (Hanson and Roberson, 26). In preparation for infection and weekly during the experiment, the hair was clipped on the dorsal tail head and a commercial depilatory agent applied to the areas to remove the remaining hair. After being tranquilized with 0.3 ml of a Ketamine Hydrochloride/Rompun (Xylazine) solution (16.7 mg/ml and 5.0 mg/ml respectively), each hamster was inoculated via the intradermal route with approximately 1.5 X 10^7 promastigotes of L. (V.) braziliensis near the base of the tail using a 0.25 ml glass syringe equipped with a 30 gauge X 1/2" needle. Each experimental group consisted of six hamsters. Initial body weights were obtained and administration of therapy, via various routes dependant on the nature of the compound, was initiated on Day 19 postinfection, and continued through Day 22 postinfection. Glucantime was included at two dosage levels (832 and 208 total mg/Sb/kg) as the reference compound, and a group of six hamsters received vehicle only (HEC-Tween). Test compounds were administered generally at 320 and 52 total mg/kg.

Lesion area of each experimental hamster was determined one week after completion of treatment with the aid of a template made at WRAIR and calibrated according to the

formula $r_1 r_2 \Pi$ where r_1 is the major radius of the lesion and r_2 is the minor radius (Wilson, et al., 27). The mean lesion area of each experimental group was obtained and the percent suppression of lesion size calculated by comparing the mean lesion area of each treated group with that of the group receiving vehicle only using a computer program and a Gateway 2000 microcomputer. The computer program performs linear and non-linear regression analysis and calculates an SD_{50} for each active compound using both analyses. The SD_{50} obtained from the non-linear analyses is used for a rough comparison of the relative efficacies of the test compounds as well as the relative efficacy of each test compound with that of the reference compound, Glucantime. The linear regression analysis is performed for comparison with the non-linear analysis.

II. Infectivity of Patient Cultures for Laboratory Animals and Verification of the Presence of Leishmania.

A variety of cultures from patients suspected of leishmanial infection were periodically sent to this laboratory from WRAIR for study of infectivity for laboratory animals. When received, each culture was examined microscopically for the presence of flagellates and a portion of each culture was subinoculated into Schneider's Drosophilia medium (Hendricks et al., 25). Each of the subinoculated cultures were checked microscopically weekly for a four week period. Positive cultures were transferred to fresh media while negative cultures were terminated after the four week period. The remainder of the culture received from WRAIR was inoculated into hamsters and/or mice as instructed by WRAIR officials. Essentially the same procedures for injection of laboratory animals and the monitoring for the presence of infection were used in this aspect of the work as was described in the preceding section for drug studies. Following is a brief summary of these procedures.

A. Hamsters

Male golden hamsters (Mesocricetus auratus) 40-80 gm were used. Patient cultures were injected into tranquilized hamsters via the intracardial, interperitoneal, or intradermal route as per WRAIR instructions. Groups of 4 hamsters per culture were used.

Hamsters receiving injections via the intracardial or interperitoneal route were each given 0.1-0.2 ml of the desired culture. Each group was monitored daily for clinical signs and/or death for a period of 6-12 months. If death occurred during this time, impressions were made of the liver, spleen, and bone marrow and cultures seeded with

ground spleen homogenate. At the end of the observation period, all surviving hamsters were killed with CO_2 and spleens were removed and ground in Schneider's Drosophilia media using a Ten Broeck tissue grinder. The spleen homogenates from each hamster from the group were then combined and a sample inoculated into a single culture flask containing Schneider's Drosophilia media, 20% inactivated Fetal Bovine Serum (GibcoBRL, Life Technologies, Grand Island, N.Y.) and 1.0% gentamycin solution (Sigma Chemical Co., St. Louis, MO). These pooled spleen cultures were then shipped to WRAIR for their perusal.

Prior to intradermal inoculations, the hair of hamsters was clipped on the dorsal tail head. Injections of 0.05-0.1 ml of the desired culture was injected into tranquilized hamsters at the base of the tail. Weekly thereafter, hair was removed from the tail head of each hamster using a commercial depilatory agent (Nair, Carter Products, Carter-Wallace, Inc., New York, NY). Hamsters were observed daily for lesion development or other clinical signs and/or death for a period of 6-12 months. When lesions appeared, photographs were taken of all lesions, a hamster with a representative lesion was killed with CO_2, the cutaneous lesion removed, touch preparations of the lesion were made on microscope slides, and the lesion ground in Schneider's Drosophila media, 20% inactivated Fetal Bovine Serum and 1% gentamycin solution. Photographs of all lesions and microscope slides with lesion impressions were forwarded to WRAIR as they were obtained.

Cultures obtained were passaged weekly for 3-4 weeks and a culture was shipped to WRAIR. A duplicate culture from each hamster lesion was passaged weekly thereafter in this laboratory for an additional 17-30 weeks dependant on the growth of the culture and freezing down time of the WRAIR laboratory. When advised that WRAIR had successful frozen isolates, the cultures were discarded. If groups of hamsters remained negative for lesion growth for a period of six months, the group was terminated by CO_2 asphyxiation.

B. Mice

Two to four C_3H mice (Harlan Sprague Dawley, Inc., Indianapolis, Indiana) 15-20 gm were injected via the intraperitoneal route with 0.1-0.2 ml of selected cultures. Mice were observed daily for clinical signs and/or death. On Day 21 postinoculation, fresh preparations were made from tail blood and examined microscopically for the presence of parasites. Results were recorded. Mice were held and observed for 6-12 months postinoculation and then killed with CO_2, kidneys removed, tissue samples taken from each kidney, all samples placed in a Ten Broeck tissue grinder,

and ground in Schneider's Drosophilia medium. The kidney homogenate from the group was inoculated into a single culture flask containing Schneider's Drosophilia media, 20% inactivated Fetal Bovine Serum, and 1.0% gentamycin solution. These pooled mouse kidney cultures were then shipped to WRAIR for their perusal.

Results and Discussion

A total of 16 new compounds which were selected by officials at WRAIR and forwarded to this laboratory were studied for antileishmanial efficacy against visceral leishmaniasis caused by L. L. donovani in the hamster (Table I). Represented among these compounds were some natural products and some compounds known to be efficacious against other diseases. Although testing was done using several dosage levels as well as various routes of administration, as can be seen from Table I, none of these compounds were noted to have any efficacy against visceral leishmaniasis in the hamster at any dosage level studied or when administered via any route. Several of the compounds noted to be inactive against L. L. donovani in the hamster were toxic to the host, often resulting in death (Table I).

The data obtained from this group of compounds indicates that none have any potential for further study in the chemotherapy of visceral leishmaniasis.

Three of the same compounds studied for efficacy against L. L. donovani were tested for activity against cutaneous leishmaniasis caused by L. V. panamensis in hamsters (Table II). Although administration was done by different routes and at different dosage levels, none of these compounds had any efficacy against L. V. panamensis regardless of dosage level or route of administration used. As noted when studying these same compounds against L. L. donovani in hamsters, some of these were toxic to the host.

The data obtained from the studies of these compounds indicates that they have very little promise for further consideration in the chemotherapy of cutaneous leishmaniasis.

A total of 62 cultures obtained by WRAIR from 44 patients were received by this laboratory for injection into hamsters and/or mice to assist in the confirmation of the presence or absence of protozoan blood parasites, to assist in determination of the type of protozoan parasite present, and to determine the susceptibility of laboratory animals (especially hamsters) to the parasites. A summary of the results is presented in Table III.

Sixteen of the cultures received produced cutaneous lesions in hamsters generally ranging from 5 to 15 mm in diameter. Microscopic examination of microscope slide impressions of these lesions revealed the presence of amastigotes of Leishmania. When the lesions were homogenized and cultured in Schneider's Drosophilia culture

medium, promastigote stages of Leishmania were observed. These observations confirm the presence of cutaneous species of Leishmania in 16 of the original cultures received from WRAIR. Furthermore, hamsters are susceptible to these Leishmania and the clinical disease observed in hamsters is similar to that observed in some established hamster-Leishmania model systems that are being used currently for chemotherapy studies (Hanson, et al., 12). Thus a laboratory animal model system is now in place that can be used to perform drug sensitivity studies with these recent isolates of cutaneous Leishmania as well as for experiments to develop new antileishmanial drugs for any of these that may be resistant to drugs currently being used for treatment.

Injection of samples of selected cultures into hamsters via the intracardiac route was done to determine whether the cultures contained any visceral species of Leishmania since this is the preferred route of infection of laboratory animals with these species (23). As can be seen from Table III, no hamsters have yet become infected with Leishmania which were injected via this route with any of the cultures selected. These experiments are still in progress and conclusions regarding the presence or absence of visceral Leishmania in the cultures cannot be drawn at this time.

Injection of samples of selected cultures into mice via the intraperitoneal route was done primarily to determine whether the cultures contained any parasites of the genus Trypanosoma. None of the mice injected with any of the cultures selected developed demonstrable infections with Trypanosoma.

Biochemical characterization (typing) of these Leishmania in other laboratories (Jackson, personal communication) has verified that the cultures received by us and have produced cutaneous lesions in hamsters in our laboratory include Leishmania Viannia panamensis, L. V. braziliensis, and L. L. mexicana. Results of the typing studies also indicated that no difference can be detected between the parasites cultured from hamster lesions and those in the original cultures thus enhancing the usefulness of these strains of Leishmania in future chemotherapy and other types of studies.

Conclusions

1. The probability of identifying new compounds with antileishmanial activity and little or no host toxicity is low.

2. The lack of success encountered in this work in identifying new, effective antileishmanial drugs emphasizes the need for continued studies of possible potentially active new compounds.

3. Leishmanial cutaneous lesion development in golden hamsters is very helpful, along with other procedures, for the confirmation of the presence of Leishmania in cultures obtained by WRAIR from patients with suspected infections with these parasites.

4. The infection of golden hamsters with several species of Leishmania of human origin (identified in other laboratories by biochemical typing as Leishmania Viannia panamensis, Leishmania Leishmania mexicana, and Leishmania Viannia braziliensis) resulted in clinical disease similar to that seen in human beings. Thus a laboratory animal model is now in place for the performance of drug sensitivity studies on these new isolates of Leishmania, some of which apparently had various degrees of susceptibility to current therapeutic drugs.

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Appendix

Table I. Summary of the suppressive activity of selected compounds against L. donovani in the golden hamster.

Bottle Number	Route	Dosages (TMK)	Percent Suppression
BN50736	PO	832,208,52	-6,-11,-76
	PO	832,208,52	17,0,-1
BN52829	IM	320,52	<-29, <-15
	PO	320,52	-12, <-9
BN55339	PO	320,52	60,44
	IP	320,52	46,44*
BK73690	PO	104,26	0,-5
BK94984	PO	208,52,13	27,12,0
BL00049	PO	208,52,13	3,-7,3
	SQ	208,52,13	15,8,-5
BL03899	PO	208,52,13	-5,-9,1
	SQ	208,52,13	ND**,5,-8
BL05357	PO	208,52,13	21,19,-7
BL05366	PO	208,52,13	21,-7,-7
	SQ	208,52,13	34,8,9
BL07637	PO	208,52,13	26,-15,-15
	SQ	208,52,13	10,8,-16**
BL07646	PO	208,52,13	5,16,8
	IM	208,52,13	49,31,16***
BL08223	PO	208,52,13	12,1,9
	IM	208,52,13	27,39,17***
BL09033	PO	208,52,13	-9,-2,-2
	IM	208,52,13	45,17,11***
BL09462	PO	208,52,13	13,25,16
BL11131	PO	208,52,13	16,29,3
ZP55109	PO	208,52,13	17,17,19
	IM	208,52,13	27,39,17***

Table I. (continued)

PO: per os
IM: intramuscular
IP: intraperitoneal
SQ: subcutaneous
* Toxic as indicated by death and greater than 15% loss of body weight.
** ND: Not done. All hamsters receiving this dosage level died prior to completion of treatment.
*** Toxic as indicated by death of 1-5 hamsters prior to completion of treatment.

Table II. Summary of the suppressive activity of selected compounds against L. Viannia braziliensis in the golden hamster.

Bottle Number	Route	Dosages (TMK)	Percent Suppression
BN50736	PO	832,208,52	-47.-58,-44*
BN52829	IM	320,52	32,30*
	PO	320,52	8,5*
BN55339	PO	320,52	-9,5
	IP	320,52	51,37

PO: per os
IM: intramuscular
IP: intraperitoneal
* Toxic as indicated by 15% loss of body weight or death

Table III. Laboratory animal confirmation of the presence of *Leishmania* from cultures obtained by WRAIR from patients with suspected infections.

Culture Designation	No. of Cultures	No. Positive#/No. Injected			
		Hamsters			Mice
		IC*	IP*	ID*	IP*
KT-1944	1	0/4			0/2
MG-1108	1	0/4			0/3
MG-0706	1	0/4		0/4	0/3
DW-4351	1	0/4	0/4		0/4
IW-7867	2	0/4			
		0/4			
DP-2750	2	0/4		0/4	
		0/4		0/4	
TG-8955	1	0/4		0/4	
GL-00117	1			4/4	
WR2159	1			4/4	
TP-9111	1	0/4			
WR2158	1			4/4	
MH-6147	7			0/4	
				0/4	
				0/4	
				0/4	
				0/4	
				0/4	
				0/4	
WR2156	1			0/4	
WR2165	2			1/4	
				0/4	
WR2164	1			4/4	
WR2157	1			1/4	

Table III.
(continued)

WR2160,	2		4/4
WR2162			4/4
WR2169	2		4/4
			4/4
JJ-4003	1		0/4
WR2172H	1		1/4
JG-1464	1		0/4
PO-4048	1		0/4
WA-8771	1	0/4	0/4
RC-4977	1		0/4
MJ-9322	1		0/4
CE-2984	3		0/4
			0/4
			0/4
AR-5950	1		0/4
ES-6474	1		0/4
TM-6684	1		0/4
WR2174,	3		4/4
WR2175,			4/4
WR2176			4/4
CC-4003	1		0/4
WR2170	1		4/4
TB-4927	1		0/4**
JK-2970	1	0/4	0/4**
DB-7413	2		0/4**
			0/4**
WR2177,	2	0/4	0/2**
WR2178		0/4	0/2**
WR2180	1		0/4**
WR2179	1		0/4**
AH-7631	1		0/4**

Table III.
(continued)

LV-8134	1		0/4**
	2		0/4**
HY-5897			
			0/4**
WR2182	1		4/4
WR2183	1		0/4**
MC-5916	1	0/4	

#: Positive for Leishmania (hamsters) or Trypanosoma (mice).
* IC: Cultures injected via the intracardiac route.
IP: Cultures injected via the intraperitoneal route.
ID: Cultures injected via the intradermal route.
** As of March 31, 1997, these animals had not been observed for the entire six month period and thus could not be designated as negative until that time.